Functional Characterization of Paillotin: An Immune Peptide Regulated by the Imd Pathway with Pathogen-Specific Roles in *Drosophila* Immunity

Yao Tian[1], Xiaojing Yue[3], Renjie Jiao[4], Mark A. Hanson[1,2]*, Bruno Lemaitre[1]*

1: Global Health Institute, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

2: Centre for Ecology and Conservation, University of Exeter, Penryn, United Kingdom

3: Laboratory of Biology and Modelling of the Cell, Ecole Normale Superieure of Lyon, CNRS, Lyon, France.

4: Sino-French Hoffmann Institute, Guangzhou Medical University, Guangzhou, China

*: Equal last author: M.A. Hanson (M.Hanson@exeter.ac.uk), B.L. Lemaitre (bruno.lemaitre@epfl.ch)

Abstract

Insects, such as *Drosophila* melanogaster, rely on innate immune defenses to combat microbial threats. Antimicrobial peptides (AMPs) play an important role in limiting pathogen entry and colonization. Despite intensive research into the regulation and biochemical properties of AMPs, their exact significance in vivo has remained uncertain due to the challenges of mutating small genes. Fortunately, recent technologies have enabled the mutation of individual AMP genes, overcome previous obstacles, and opened new avenues for research. In this study, we characterized one novel host-defense peptide, Paillotin (IM18, *CG33706*), using loss-of-function mutants. Paillotin is an ancient host defense peptide of Diptera, regulated by the Imd pathway. Loss of Paillotin does not impact the activity of either the Imd or Toll pathways. Importantly, we found that *Paillotin* mutants are viable but exhibit increased susceptibility to specific infections, particularly *Providencia burhodogranariea*. Paillotin was further found to contribute synergistically to defense against *P. burhodogranariea* when combined with other AMPs. However, we did not detect direct microbicidal activity of Paillotin *in vitro* in our hands. Taken together, our findings identify Paillotin as a novel host defense peptide acting downstream of Imd signaling, advancing our understanding of the *Drosophila* antimicrobial response.

Keywords: antimicrobial peptide, host–pathogen interactions, *Drosophila*, immunity

Introduction

Insects inhabit environments rich in microbes, necessitating a robust immune system to defend against opportunistic pathogens [1]. Unlike vertebrates, insects rely solely on innate immune defense mechanisms. The conservation of innate immunity across *Metazoans* and the powerful genetics of *Drosophila melanogaster* make this insect a powerful model for studying immune responses [2–4].

Flies exhibit behavioral immunity to prevent pathogens from entering their body cavity, such as grooming and avoidance of pathogens [5,6]. Once pathogens breach this barrier, immune responses are activated in different body regions to limit growth. Local immunity occurs at initial pathogen entry sites, like the gut or trachea. Systemic immunity involves cellular and humoral responses that take place in the body cavity upon systemic infection. Cellular responses involve hemocytes (blood cells), while humoral responses involve the production of host defense peptides as well as other effectors secreted by hemocytes and the fat body, an organ analogous to the mammalian liver [1,2]. Two NF-κB signaling pathways, the Toll and Imd pathways, regulate the humoral response. The principal effectors downstream of the Toll and Imd signaling pathways consist of AMPs, which are synthesized by both the fat body and hemocytes and then secreted into the hemolymph. AMPs are positively charged and can bind to the negatively charged membranes of microorganisms, disrupting membrane integrity through pore formation [1,7]. Upon infection, their expression is markedly increased to high levels in *Drosophila*, notably *Drosomycin* and *Diptericin,* which are often used as readouts for Toll and Imd activity, respectively [8]. Besides microbe killing, AMPs have been implicated in multiple processes such as tumor suppression[9,10], microbiota control [11], and neurodegeneration [12,13]. Currently, *D. melanogaster* is known to possess eight families of inducible AMPs, including antifungal peptides like Drosomycin, Baramicin, and Metchnikowin; Cecropins (four isoforms) exhibiting both antibacterial and antifungal activities *in vitro*; and Drosocin, Attacins (four isoforms), Defensin, and Diptericins (two isoforms), primarily displaying antibacterial activity [14–21]. Additionally, the *Drosophila* genome encodes numerous other host defense peptides, such as the Toll-regulated *Daisho* genes (two genes),

Bomanins (twelve genes), peptides encoded by *Baramicin A*, and the Imd-regulated peptide Buletin (product of the *Drosocin* gene), whose antimicrobial activity *in vitro* remains to be fully demonstrated, but functional studies have indicated their importance *in vivo* for surviving microbial infections [22–26].

Functional studies using single or combined mutations have demonstrated that these host defense peptides largely account for the critical roles of the Imd and Toll pathways in host survival following infection with Gram-negative bacteria, Gram-positive bacteria, or fungi. The antibacterial peptides Diptericin, Cecropin, Attacin, Drosocin, and Buletin account for most of the Imd pathway–mediated resistance to Gram-negative bacterial infection [25,27–29]. Drosomycin, Metchnikowin, Daisho (two genes), Baramicin A mediate much of the Toll-mediated resistance to fungal infection [23,24,26,27,30] , and Bomanins (twelve genes) appear to be the most important family of Toll-regulated host defense peptides contributing to resistance against Gram-positive bacteria and fungi [22,27,31]. These host defense peptides display both additive and synergistic effects, along with a high degree of specificity, whereby individual peptides greatly contribute to survival against a defined pathogen [27,29,32]. Some immune-induced peptides regulated by Toll and Imd have regulatory functions. This includes GNBP-like3, which is involved in modulating Toll pathway activity, as well as Edin, which has been implicated in regulating hemocyte sessility [33,34].

Despite significant advances in understanding *Drosophila* host defense peptides, many peptides remain underexplored. The *Drosophila* genome encodes several other immune-induced peptides that remain poorly characterized, including notably Listericin, CG4269, and IM18 [1,35].

In this article, we have characterized the function of IM18, which we renamed Paillotin. We showed that Paillotin is an ancient host defense peptide regulated by the Imd pathway, which does not affect the regulation of the Imd or Toll pathways. Importantly, we found that *Paillotin* mutants exhibit increased susceptibility to specific infections, particularly *Providencia burhodogranariea*. Paillotin was also found to contribute synergistically to defense against *P. burhodogranariea* when combined with other AMPs, specifically Drosocin, Diptericins, and Attacins. However, we did not detect direct microbicidal activity of Paillotin *in vitro*. Taken together, our findings identify Paillotin as a novel host defense peptide

downstream of Imd signaling, advancing our understanding of the *Drosophila* antimicrobial response.

Results

Paillotin is a conserved host defense peptide in *Diptera*

A previous proteomic analysis identified an immune inducible peptide called Immune-induced Molecule 18 (IM18) based on its mass by MALDI-TOF [36]. We rename the *IM18* gene (*CG33706*) as *Paillotin* (*Pai*) from André Paillot, who in 1920 discovered the humoral immune response of insects [37,38]. The gene encoding Paillotin was identified as *CG33706* in a region annotated as polycistronic with another gene, *CG10332*, making it challenging to distinguish their expression patterns (Figure 1*a*); the *CG10332-RA* transcript is identical to the *Pai-RA* transcript and contains the full coding sequence of both *CG10332* and *Pai*. Indeed, in FlyAtlas data, *IM18* and *CG10332* are shown with identical expression levels [39]. However, the characterization of IM18 as an immune-induced peptide pointed to the existence of two distinct gene products, perhaps regulated by the distinct transcripts *Pai-RB* and *CG10332-RA.* To confirm this, we monitored the expression of either *CG10332-RA* alone or the cumulative products of *CG10332-RA* and *Pai-RB* upon septic injury with either the Gram-negative bacterium *Escherichia coli* (*E. coli*) or the Gram-positive bacterium *Micrococcus luteus* (*M. luteus*). The results showed that the mRNA encoding *Pai-RB* (Figure 2*a*, *b*) but not *CG10332-RA* was strongly induced upon septic injury (Figure 2*c*, *d*), confirming that only *Pai-RB* is immune-induced at the transcriptional level.

The *Paillotin* gene encodes a 71-residue precursor peptide composed of a signal peptide, dipeptidylpeptidase motif, and mature peptide (Figure 1*b*). To investigate the evolutionary conservation of Paillotin, we used a recursive tBLASTn approach to screen outgroup fly species [40]. Orthologues of Paillotin were identified in *Diptera* families such as *Tephritidae* and *Diopsidae*, which share a common ancestor with *D. melanogaster* approximately 126 million years ago [41] (Figure 1*b*). The C-terminal domain displayed the highest level of conservation across species, suggesting a critical role in Paillotin function. The presence of conserved features, such as the two-residue dipeptidyl peptidase cleavage site (XA/XP) and a positive

117 charge (+3.89 in *D. melanogaster*) commonly found in AMPs [24,42], supports the hypothesis
118 that Paillotin plays a key role in host defense and could have microbicidal activities. This
119 evolutionary conservation underscores Paillotin as an ancient host defense peptide in *Diptera*.
120

121 ***Paillotin* is regulated by the Imd pathway**

122 To explore the expression pattern and regulation of *Paillotin* during systemic immune
123 responses, we analyzed transcript levels in wild-type, Toll, and Imd pathway-deficient flies
124 (Figure 2). Specifically, we challenged w^{1118} (wild-type), spz^{rm7} (deficient for the Toll pathway),
125 $Relish^{E20}$ (deficient for Imd pathway) and $Relish^{E20}$, spz^{rm7} double mutant flies with either *E. coli,*
126 an inducer of the Imd pathway (Figure 2*a*), or *M. luteus,* an inducer of the Toll pathway (Figure
127 2*b*). Expression of *Paillotin* was monitored at 8h, 16h, and 24h post-infection using qRT-PCR.
128 We observed that *Paillotin* gene expression was strongly induced after both *M. luteus* and *E.*
129 *coli* infections in w^{1118} flies at all time points. However, this induction was completely abolished
130 in $Relish^{E20}$ and $Relish^{E20}$, spz^{rm7} double mutants, but not in spz^{rm7} flies, indicating *Paillotin* is
131 regulated by the Imd pathway (Figure 2*a*, *b*). Consistent with this expression profile, we found
132 multiple putative NF-κB responsive motifs within the first 200bp of promoter sequence
133 upstream of gene transcription start sites of *Paillotin* [24,43] including five motifs uniquely
134 matching the *Relish* consensus motif sequence (GGRDNNHHBS), one motif matching a
135 *Dif/Dorsal* (GGGHHNNDVH) binding site, and two κB motifs with putative matches to either
136 *Relish* and/or *Dif/Dorsal* binding sites. In contrast, no NF-κB binding motifs were identified in
137 the 250bp upstream region of *IM18-RA/CG10332-RA*, consistent with its non-immune
138 inducibility.

139 A recent proteomic analysis of *Drosophila* hemolymph following bacterial infection [44]
140 indicated that Paillotin peptide was strongly induced in the hemolymph of wild-type flies
141 infected with *M. luteus* or the Gram-negative bacterium *Pectobacterium carotovorum* (*Ecc15*),
142 and this induction was abolished in $Relish^{E20}$ flies (Figure 2*e*). Consistent with its regulation by
143 the Imd pathway, *Paillotin* was more induced upon infection with *Ecc15* than *M. luteus*. We
144 conclude that Paillotin is a novel host defense peptide regulated at the transcriptional level by
145 the Imd pathway and secreted into the hemolymph.
146

Characterization of *Paillotin* mutants

The *Drosophila* Genetic Reference Panel (DGRP) consists of a genetically diverse collection of wild-caught flies from the USA, whose genomes have been sequenced [45]. We identified two loss-of-function mutations in *Paillotin* within the DGRP (Figure 3*a*, Table S1). The first mutation (2R_19488505_DEL) is found in eleven genotypes from the DGRP, including DGRP-41, which deletes 61 nucleotides within the Paillotin mature peptide coding region, leading to a frameshift and protein truncation (referred to as $Pai^{\triangle 41}$). The second mutation (2R_19488690_SNP), found in only DGRP-370 ($Pai^{\triangle 370}$), involves a C-T transition that disrupts the *Paillotin* start codon. Collectively, 12 of the 205 DGRP lines encode a loss-of-function mutation in *Paillotin*, like previous reports on Diptericins [29,32,40]. The third mutant, referred to as $Pai^{\triangle SFHI}$, a gift from the Guangzhou Drosophila Resource Center (Figure 3*a*), was generated by CRISPR-Cas9 and involves a 20-nucleotide deletion that causes a complex frameshift, resulting in a nonsense peptide. The precise nucleotide alterations associated with each *Paillotin* mutant allele are presented in Supplementary Figure S1. All these mutants were isogenized into the w^{1118} iso Drosdel background, except for $Pai^{\triangle SFHI}$, where only the *X* chromosome was isogenized.

RT-qPCR confirmed reduced *Paillotin* gene expression in $Pai^{\triangle 41}$ and $Pai^{\triangle 370}$, while *Paillotin* expression was completely absent in $Pai^{\triangle SFHI}$ (Figure S2). We next performed a MALDI-TOF analysis of hemolymph from adult flies infected with *P. c. carotovara Ecc15*. The results revealed the absence of the 4625Da Paillotin peaks in $Pai^{\triangle 41}$, $Pai^{\triangle 370}$, and $Pai^{\triangle SFHI}$ flies (Figure 3*b*). All the mutants were viable without morphological defects.

Paillotin does not regulate the Imd and Toll pathways

Some host defense peptides, such as mammalian LL37 or beta-Defensins, have been shown to function similarly to cytokines, modulating immune system activity [46,47]. This prompted us to assess whether Paillotin could influence Toll and Imd pathway activity. To address this, we monitored the expression of *Diptericin A* (a readout of the Imd pathway) in wild-type (w^{1118}) and *Paillotin* -deficient flies ($Pai^{\triangle 41}$ and $Pai^{\triangle 370}$) at 6- and 12-hours post-infection with *Ecc15* and *Drosomycin* (a readout of the Toll pathway) at 8- and 24-hours post-infection with *M. luteus* (Figure 4*a*, *b*). The expression levels of *Diptericin A* in $Pai^{\triangle 41}$ and

Pai$^{\triangle 370}$ mutants were comparable to those in wild-type flies at both time points, indicating no effect of *Paillotin* on Imd pathway activity. Similarly, the expression of *Drosomycin* in *Pai*$^{\triangle 41}$ and *Pai*$^{\triangle 370}$ mutants showed no significant difference compared to w^{1118}, suggesting that *Paillotin* is not involved in regulating the Toll pathway. Together, these results demonstrate that *Paillotin* is not a regulator of the Imd or Toll pathways.

***Paillotin* mutant flies are susceptible to *P. burhodogranariea* infection**

Recent studies have demonstrated that the deletion of a single AMP gene can significantly increase susceptibility to specific pathogens. For instance, the *Diptericin A* mutation leads to marked susceptibility to *Providencia rettgeri*, while the *Diptericin B* mutation greatly reduces resistance to *Acetobacter sp.* [29,32]. To investigate the role of Paillotin in resistance to infections, we examined its contribution against a range of Gram-negative, Gram-positive bacteria, and fungi. In these experiments, we compared the survival of *Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$ flies to that of the wild-type (w^{1118}), *Relish*^{E20}, *Bom*$^{\triangle 55}$, and *spz*^{rm7} immune-deficient mutants. Firstly, we did not detect any susceptibility of *Paillotin* mutants to clean injury, indicating that this peptide does not play a critical role in wound healing (Figure S3). Regarding Gram-negative bacteria, there was no statistically significant difference in survival between wild-type and *Paillotin* mutants following infections with *P. rettgeri*, *Ecc15*, or *Enterobacter cloacae* (Figure S4*a-c*). However, all three *Paillotin* mutant flies (*Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, *Pai*$^{\triangle SFHI}$) exhibited a modest but significant increase in susceptibility to septic injury with *Providencia burhodogranariea* (*P. burhodogranariea*) (Figure 5*a*). This susceptibility was less marked than *Relish*^{E20} flies, indicating that other peptides downstream of the Imd pathway contribute to survival upon *P. burhodogranariea* infection. In addition, we observed higher *P. burhodogranariea* bacterial load in *Paillotin* mutants compared to wild-type, indicating that Paillotin suppresses bacterial growth (Figure 5*b*). For Gram-positive bacteria, *Paillotin* mutants showed no consistent increased susceptibility to *Enterococcus faecalis* (Figure 5*c*), *Bacillus subtilis*, *Listeria monocytogenes*, or *Staphylococcus aureus* infections (Figure S4*d-f*). Similarly, fungal infections with *Beauveria bassiana* (Figure 5*d*), *Aspergillus fumigatus, Metarhizium anisopliae*, or *Metarhizium robertsii* did not reveal any increased susceptibility of *Paillotin* mutants compared to wild-type (Figure S4*g-i*).

207

208 **No *in vitro* activity of Paillotin peptide**

209 Cecropins were the first inducible AMPs identified in moths [14]. *In vitro* studies have
210 shown that Cecropins are highly effective against a wide range of Gram-negative bacteria at
211 concentrations lower than those induced in insects during infection (25–50 μM) [48]. To
212 investigate the antimicrobial potential of Paillotin *in vitro*, we produced the peptide and
213 evaluated its activity against *P. burhodogranariea* at 100μM alone or in combination with
214 sublethal concentrations of Cecropin A. However, Paillotin exhibited no detectable
215 microbicidal activity against *P. burhodogranariea in vitro* (Figure S5*a*). It also failed to inhibit
216 the growth of other Gram-negative bacteria, including *Ecc15* and *E. coli* (Figure S5*b*, *c*). Thus,
217 we found no activity of Paillotin *in vitro* under these conditions.

218

219 **Paillotin synergistically contributes to the defense against *P. burhodogranariea***
220 **with other AMPs.**

221 Recent studies have demonstrated synergistic or additive cooperation among AMPs,
222 notably against *P. burhodogranaria*. While single mutations in *Drosocin*, *Attacins*, or
223 *Diptericins* have minimal overall effect, a compound mutant referred to as the "*Group B"* AMP
224 mutant lacking *Drosocin*, *Attacins A, B,* and *C*, and *Diptericins A* and *B*, displays marked
225 susceptibility to *P. burhodogranariea* infection [27]. To investigate potential interactions
226 between *Paillotin* and these AMPs, we used genetic crosses to combine the *Paillotin* mutation
227 with *Group B* mutants. We initially infected *Group B* mutants, *Paillotin* mutants, and *Group B,*
228 *Paillotin* compound mutants with *P. burhodogranariea*. However, we observed no synergistic
229 or additive effects, as the introduction of *Paillotin* mutations into *Group B* mutants did not
230 further increase susceptibility. This lack of an observable effect may be due to the already high
231 susceptibility of *Group B* AMP mutants to this bacterium, even at a low inoculum (OD_{600}=0.1),
232 potentially masking any additional contribution from the *Paillotin* mutation (Figure 6, dashed
233 curves). To circumvent this, we assessed the impact of *Paillotin* homozygous deficiency in flies
234 heterozygous for *Group B* AMP mutations (*Group B*/+), which depresses the inducibility of
235 AMPs compared to homozygotes [27]. Notably, in this sensitized genetic background, the
236 absence of *Paillotin*, regardless of the allele used, significantly increased susceptibility of

237 *Group B*/+ flies to *P. burhodogranariea* (Figure 6*a-c*, solid curves). This co-occurring loss of
238 resistance appears to be primarily synergistic (Mutant, Cox Hazard Ratio (HR), *p*-value:
239 *Paillotin*, HR =-0.17, *p* =0.01; *Group B*, HR =-1.06, *p*<0.001; *Paillotin***Group B*, HR =-0.15,
240 *p*=0.02). In addition, a marginally significant trend consistent with a synergistic effect between
241 *Paillotin* and *Group B* mutants was also seen for infections with the Gram-negative bacteria
242 *Providencia rustigianii* and *Providencia vermicola*, but not *E. coli* (Figure S6). This supports
243 the notion that Paillotin confers a fairly specific resistance to *P. burhodogranariea*, perhaps also
244 extending to other *Providencia* species.
245

246 **Overexpression of Paillotin restores resistance in Paillotin-deficient flies against *P.***
247 ***burhodogranariea***

248 The observation that three distinct null mutations in *Paillotin* are associated with a higher
249 susceptibility and the synergistic effect between Paillotin and other AMPs against infection
250 provide strong evidence for a role of this host defense peptide in resisting *P. burhodogranariea*.
251 To reinforce this finding, we performed a genetic rescue experiment by combining the *Paillotin*
252 mutation with a *UAS-Pai* transgene. We initially observed that overexpression of *Paillotin* in a
253 wild-type background was not sufficient to enhance resistance to *P. burhodogranariea* (Figure
254 S7). In contrast, overexpression of *Paillotin* using either the *C564-Gal4* (fat body-specific) or
255 *Actin-Gal4* (ubiquitous) driver restored pathogen resistance to near wild-type levels in the
256 $Pai^{\triangle 41}$ (Figure 7*a*) and $Pai^{\triangle 370}$ (Figure 7*b*) mutant backgrounds. The observation that three
257 distinct *Pai* alleles confer susceptibility, together with the results of the rescue experiment,
258 collectively demonstrates that Paillotin acts as a pathogen-specific host defense peptide
259 essential for restricting *P. burhodogranariea* proliferation.

260 The Imd and Toll signaling pathways are essential for insect immunity, with double
261 pathway-deficient flies exhibiting extreme susceptibility to infection due to their inability to
262 upregulate hundreds of immune effector genes, particularly AMPs [49]. Previous work has
263 demonstrated that transgenic overexpression of individual AMPs in some cases is sufficient to
264 restore resistance in *Imd, Toll-deficient* flies [50]. Building on this paradigm, we generated flies
265 overexpressing *Paillotin* using *Actin-Gal4* or *C564-Gal4* drivers in $Relish^{E20}$ mutant flies
266 (Figure 7*c*). However, *Paillotin* overexpression failed to confer protection against *P.*

burhodogranariea infection. While we cannot exclude that Paillotin was not expressed at a sufficient level to overcome the extreme susceptibility of *Relish* mutants, this suggests that *Paillotin* alone is insufficient and the host requires the presence of other AMPs for the defense response to function effectively, consistent with the synergistic interactions observed with *Group B/+* heterozygous mutants.

Discussion

In this study, we characterized Paillotin, an evolutionarily conserved host-defense peptide regulated by the Imd pathway. We first demonstrated that the *Paillotin-RB* transcript is highly distinct from *CG10332-RA*, encoding the secreted immune peptide Paillotin. Paillotin displays several characteristics of an antibacterial peptide, being small, cationic, encoding a dipeptidyl peptidase cleavage site common to many AMPs, and being strongly induced and regulated by the Imd pathway [51]. Phylogenetic analysis reveals the conservation of this peptide among *Diptera*, with a high conservation of the C-terminal domain, suggesting the core Paillotin peptide can play a role in the immune response against the diversity of pathogens that these groups of insects are exposed to. Interestingly, there is a relatively high frequency of loss-of-function mutation segregating in wild *Drosophila* populations (Table S1), suggesting Paillotin is not integral to the generic host defense. This observation is consistent with our results showing that the *Paillotin* mutation affects defense against only a few bacteria out of the pathogens tested, suggesting it is dispensable in defense against many microbes, but useful against some microbes like *Providencia sp.* whose presence is stochastic in wild-caught flies [52].

We observed that all three *Paillotin* mutants are viable and do not impact the overall activity of the Imd or Toll pathways. However, these loss-of-function mutations in *Paillotin* increased the susceptibility of flies to *P. burhodogranariea* infection, and perhaps other moderately virulent *Providencia* species, without altering survival to other tested bacterial or fungal species. Furthermore, the *Paillotin* mutation synergistically enhances susceptibility to *P. burhodogranariea* in flies heterozygous for mutations in *Drosocin*, *Diptericins A and B*, and

295 *Attacins A, B, and C.* Taken together, the consistent susceptibility of three independent *Paillotin*
296 alleles to *P. burhodogranariea*, accompanied by elevated bacterial loads, the resistance of
297 *Paillotin*-deficient flies to other tested pathogens, and the successful rescue of the phenotype
298 by overexpression, all provide strong evidence that *Paillotin* functions as a host defense peptide
299 specifically effective against this bacterium. A high degree of specificity has been observed for
300 other host defense peptides, whose inactivation frequently causes pathogen-specific immune
301 deficiency. This has been illustrated not only for Diptericin A and B as described previously
302 [29,32], but also for Drosocin, which provides significant protection against *E. cloacae* [25,27].
303 The existence of such specificity between a defined host defense peptide and particular
304 pathogens suggests that these genes have been evolutionarily maintained under selective
305 pressure imposed by specific microbes encountered in natural environments [53].

306 Previous studies have shown that resistance to *P. burhodogranariea* involves multiple
307 antimicrobial peptides, including Buletin (encoded by the *Drosocin* gene), Diptericins A and B,
308 and Attacins A, B, C, and D. While single AMP mutations in many genes do not greatly impact
309 survival following infection with *P. burhodogranariea*, compound mutants lacking *Drosocin*,
310 *Diptericins* and *Attacins* exhibit increased susceptibility, suggesting the presence of a
311 synergistic effect among these peptides [27]. More recently, the absence of Buletin—a peptide
312 generated by furin cleavage of the Drosocin precursor—results in notable survival differences
313 following *P. burhodogranariea* infection [25]. Thus, host resistance to *P. burhodogranariea* is
314 mediated by the collective action of multiple host defense peptides, and our study now adds
315 Paillotin to this list. Future studies may further reveal additional roles of Paillotin, either alone
316 or in combination with other host defense peptides, in resistance to other pathogens.

317 We were unable to detect any direct *in vitro* microbicidal activity of Paillotin against *P.*
318 *burhodogranariea* and other bacteria, either alone or in combination with Cecropin. Several
319 factors could explain this lack of detectable activity: i) Paillotin may depend on host-specific
320 factors for its function, such as the requirement for a cofactor or modulation by environmental
321 cues (e.g., pH or ionic strength), or ii) synergistic interactions with other immune molecules
322 that are only present *in vivo*. Similar synergistic mechanisms have been observed in other
323 systems. For instance, in *Galleria mellonella* (the greater wax moth), an anionic antimicrobial

324 peptide (anionic peptide 2) enhances lysozyme-mediated membrane perforation, facilitating the
325 elimination of *E. coli*. [54]. Furthermore, AMPs often enhance each other's efficacy through
326 complementary mechanisms of action [21,55]. In addition, recent studies have pointed to the
327 role of host defense peptides in resilience mechanisms, including tolerance to host immune
328 molecules [44] and pathogen toxins [26,31]. However, we did not detect a role for Paillotin in
329 resilience to the fungal toxins Destruxin A or Verruculogen (Figure S8). Interestingly, in
330 contrast to recent studies [26,31], we did not detect a susceptibility of Toll-deficient spz^{rm7} flies
331 to these two toxins (Figure S8). Of note, the description of DIMs has been done largely by
332 collecting peptides in Trifluoroacetic acid, which is known to modify peptides by, for example,
333 deamidating NG motifs [24,56]. It is possible that the endogenous peptide products of the host
334 immune response differ from the peptides observed in proteomic analysis and synthetic
335 peptides used in recent studies, in unknown ways. Early studies on AMPs typically found
336 striking activity *in vitro* using peptide extracts purified from endogenous hemolymph or cell
337 line supernatant [14,17,19,21,57]. Future studies may benefit from a return to such approaches,
338 including gel overlay experiments that can simplify the isolation of the activity of single
339 peptides from endogenous samples [58] or collecting crude peptide extracts from
340 overexpressing cells or larval hemolymph. However, future studies might also explore whether
341 Paillotin protects against virulence factors specific to *P. burhodogranariea*. For now, despite
342 displaying many features of AMPs, we cannot conclusively classify Paillotin as a bona fide
343 AMP. It should be classified as a host defense peptide until its molecular mode of action is fully
344 determined.

345 The use of compound module-deficient mutants has revealed the major role of the Imd
346 pathway, and to a lesser extent, melanization, but not the cellular response to survive *P.*
347 *burhodogranariea* infection [59]. Since Paillotin does not affect Toll and Imd pathway activities,
348 we cannot exclude a role of this peptide in the melanization reaction. However, this role would
349 have to be subtle as *Paillotin* mutants display a wild-type blackening reaction upon clean injury
350 (Figure S9*a*). Previous studies have shown that flies lacking 14 AMPs exhibit increased
351 microbiota abundance and altered composition in old flies, suggesting that AMPs are involved
352 not only in pathogen resistance but also in shaping the environmental microbiota [11]. However,

353 we did not detect any significant differences in gut microbiota load between wild-type flies and
354 *Paillotin* mutants (Figure S9*b*).

355 To conclude, our study characterizes a new *Drosophila* host defense peptide, Paillotin,
356 which plays a specific role against *P. burhodogranariea*. IM18/Paillotin is the last gene
357 encoding an immune molecule identified by MALDI-TOF in the 1998 study [36] to be
358 characterized, following the successful characterization of Bomanins [30], Daisho [23], Buletin
359 [25], and Baramicin [24,26]. Together, these studies and the present article provide important
360 insights into the *Drosophila* humoral immune response, revealing a more complex picture than
361 initially anticipated. Using the power of *Drosophila* genetics to dissect how immune effectors
362 individually or collectively contribute to host defense provides a general principle on the
363 architecture of innate immune systems.

364 Materials and Methods

365 Sequence Comparisons

366 Genomic sequences were obtained from GenBank default reference assemblies [60], and
367 DGRP sequence data from the DGRP database [45] (http://dgrp2.gnets.ncsu.edu/). Sequence
368 comparisons and alignment figures were prepared using Geneious R10 [61]. Putative NF-κB
369 binding sites were annotated using the *Relish* motif "GGRDNNHHBS" as described in Copley
370 et al [43], along with a manually curated amalgam motif "GGGHHNNDVH" described in
371 Hanson et al [24].

372 Paillotin annotation across species was performed as described in Hanson et al [40]. In
373 brief, a recursive tBLASTn approach was used with an artificially high E-value (E < 1000) to
374 compensate for the short length of the AMP query sequence. All the best BLAST hits were
375 assessed manually. If no Paillotin was found in a genome or lineage, only the conserved C-
376 terminus was used as a query in a repeat attempt (improving sensitivity). No *Paillotin*
377 candidates were discovered outside *Brachycera*, which included screens of the sequenced
378 nematoceran genomes of various mosquitoes, *Chironomus riparius, Clogmia albipunctata,*
379 *Tipula unca, Lutzomyia longipalpis, Phlebotomus papatasi, Sylvicola cinctus,* and *Bibio marci*.

Fly Strains

Flies were maintained at 25°C on standard cornmeal-agar media. w^{1118} flies were used as wild-type controls. The positive controls for infection assays for Gram-positive and Gram-negative infections were spz^{rm7} and $Relish^{E20}$. The $Pai^{\Delta 41}$ and $Pai^{\Delta 370}$ mutations are loss-of-function variants in the *IM18* gene within the DGRP, and $Pai^{\Delta SFHI}$ was kindly provided by Guangzhou Drosophila Resource Center, generated via CRISPR/Cas9. The guide RNAs that targeted *Pai* were cloned into the pUAST-attB plasmids before injecting into the *y [1] M {RFP [3xP3.PB] GFP[E.3xP3] =vas-int.Dm}ZH-2A w [*]; M{3xP3-RFP.attP} ZH-86Fb* (BL24749) to get gRNA transgenic flies. The gRNA flies were then crossed with flies containing nos-Cas9 to get mutants. The *UAS-Pai* constructs were prepared using the TOPO pENTR entry vector and cloned into the pTW system. Genetic variants were isogenized into the DrosDel isogenic background over seven generations as previously described [62] using the following primers that are specific to an indel in the *Pai* locus common to both the $Pai^{\Delta 41}$ and $Pai^{\Delta 370}$, but which is absent in the DrosDel background: Pai_41F (ACGTACGGTCAAAAGCAACTACTTCG) and Pai_R3mut1 (CCAATCCCAAGGGCACTCGTATATG), amplified with a 66°C annealing temperature. All the fly strains used in the paper are listed in Table S2.

MALDI-TOF Proteomic Analysis

Hemolymph samples were collected from *Ecc15*-infected flies (OD600 = 200, 24 hours post-infection, 50 flies/sample) using a nanoinjector (Nanoject, Drummond Scientific, Broomall, PA) for MALDI-TOF analysis. Trifluoroacetic acid (0.1% TFA) was added to the hemolymph before mixing with the acetonitrile matrix. Samples are processed by SPE (Zip-Tip C18) before MALDI. Peaks were identified based on corresponding m/z values from previous studies [24,63] in ISIC's Mass Spectrometry facility of EPFL. Spectra were visualized using Origin software, with figures prepared in Prism 10.

Gene Expression Analysis

Total RNA was extracted from pooled samples of ten flies using TRIzol. cDNA was synthesized using Takara Reverse Transcriptase. Quantitative PCR (qPCR) was performed using PowerUP SYBR Green Master Mix with the primers. Results represent the average of

408 three independent experiments. Primers used in this study were:

409 *Drosomycin*-F 5'- CGTGAGAACCTTTTCCAATATGAT-3'

410 *Drosomycin*-R 5'- TCCCAGGACCACCAGCAT-3'

411 *Diptericin A*-F: 5'-GCTGCGCAATCGCTTCTACT-3'

412 *Diptericin A*-R:5'-TGGTGGAGTGGGCTTCATG-3'

413 *Rpl32*-F: 5'-GACGCTTCAAGGGACAGTATCTG-3'

414 *Rpl32*-R: 5'-AAACGCGGTTCTGCATGAG-3'

415 *Pai*-F: 5'-GATCCGTTTCCTGGTCCAGTGTG-3'

416 *Pai*-R: 5'-CCATGAAGCTGATCGCATTGTGC-3'

417 *CG10332*-F: 5'-TCAGAGGCTGCACCATCAGAAGT-3'

418 *CG10332*-R: 5'-CCGCTTCAGAGGCTGCACC-3'

419 **Microbial Culturing Conditions**

420 *Micrococcus luteus*, *Erwinia carotovora carotovora (Ecc15)*, and *Providencia*
421 *burhodogranariea* strain B were cultured overnight in LB broth at 29°C. *Enterobacter cloacae*,
422 *Escherichia coli* strain 1106, *Providencia rustigianii*, *Providencia vermicola, Providencia*
423 *rettgeri,* and *Staphylococcus aureus* were cultured overnight in LB at 37°C. *Enterococcus*
424 *faecalis*, *Bacillus subtilis,* and *Listeria monocytogenes* were cultured overnight in BHI at 37°C.
425 *Aspergillus fumigatus* was grown on malt agar plates at 37℃, and *Metarhizium anisopliae* and
426 *Metarhizium robertisii* were cultured on malt agar plates at 25°C for around two weeks until
427 sporulation was observed. *Beauveria bassiana* strain R444 commercial spores were produced
428 by Andermatt Biocontrol, products: BB-PROTEC. Bacteria were pelleted at 4000 rpm for 15
429 minutes at 4°C, resuspended in PBS, and diluted to the desired OD600 value indicated in the
430 figure legends. The fungal material was filtered through glass wool using 0.05% Tween-80 to
431 collect spores and diluted to the desired concentration (in spores/ml). All microbial strains used
432 in this study are listed in Table S3.

433 **Microbiota Load Analysis**

434 Flies were collected 30 hours post-infection and surface-sterilized with 70% ethanol.
435 Whole-fly homogenization was performed in LB broth using a bead beater at 7200 rpm for 30
436 seconds. Serial dilutions of the homogenates were plated (100 μL per plate) on LB agar and
437 incubated at 29 °C for 24 hours. Colony-forming units (CFUs) were then manually counted.

438 For gut microbiota load analysis, 18-day-old conventionally reared flies were collected
439 and processed using the same procedure, except that MRS medium was used for culturing.
440 Plates were incubated at 29 °C for 48 hours prior to CFU quantification.

441 ***In Vitro* Antibacterial Activity**

442 The Paillotin peptide (45 residues) was synthesized by GenicBio with >95% purity, and
443 Cecropin A (from silk moth) was obtained from Sigma-Aldrich (≥97% purity). Peptides were
444 dissolved in water. Bacteria were cultured overnight and diluted to OD600 = 0.005 in LB broth,
445 then incubated for 2 hours. Paillotin peptide and Cecropin A were diluted in LB broth to the
446 desired concentration. Bacterial cultures (1 μL) were added to peptide solutions in a 96-well
447 plate, and OD600 was recorded every 10 minutes using a TECAN plate reader.

448 **Survival Assays**

449 Male flies (3-5 days old) were used for survival assays. The thorax of each fly was pricked
450 with a needle (diameter ~0.2mm) dipped in a bacterial suspension. For the fungi infection, flies
451 were rolled in the plates of *A. fumigatus* or the commercial spores of *B. bassiana,* or were
452 dipped into a spore solution of *M. anisopliae* or *M. robertisii*. Mortality was recorded daily.
453 Infected flies were maintained at 29°C or 25°C and transferred to fresh vials three times per
454 week.

455 **Toxin Injection**

456 Destruxin A (Sigma) was dissolved in high-purity dimethyl sulfoxide (DMSO) and
457 subsequently diluted with PBS to a final concentration of 8 mM. A volume of 9.2 nL of this
458 solution was administered to flies using the Nanoject III microinjector (Drummond)[26].
459 Verruculogen (Abcam) was prepared in DMSO at a concentration of 1 mg/mL, and 4.6 nL of

this solution was injected into the flies[31].

Wounding experiment

Clean injury was performed with a needle sterilized (diameter ~0.1mm) with an EtOH and PBS wash. Cuticle blackening was categorized into normal, weak, or no melanization 24h post-pricking at the injury site.

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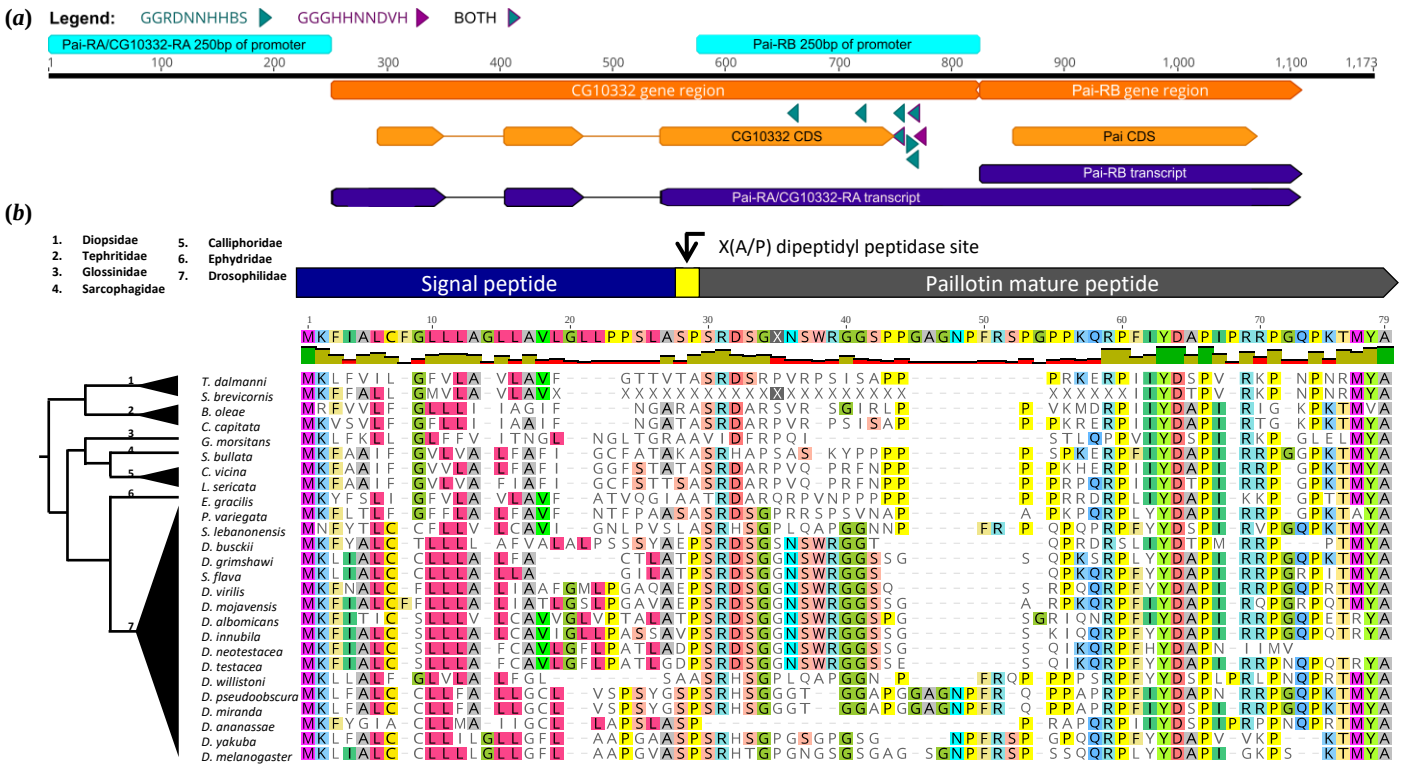
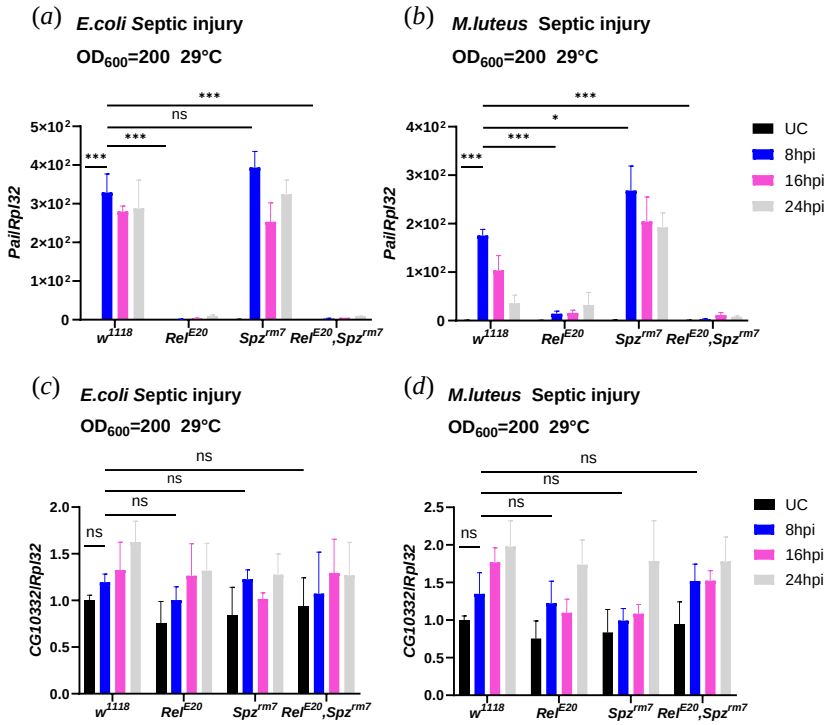


Figure 1: The *Paillotin* gene structure and conservation. (*a*) Schematic representation of the *Paillotin* (*Pai*) locus. The *Pai-RB* transcript is annotated as polycistronic, sharing a promoter and transcript with *CG10332* (*CG10332-RA*), indicating both gene CDS regions are co-transcribed. Promoter regions (250 bp upstream) for *Pai-RB* and *CG10332-RA* are shown in cyan. Colored triangles indicate putative NF-κB binding motifs: *Relish*-responsive motifs (GGRDNNHHBS) in teal, *Dif/Dorsal*-responsive motifs (GGGHNNNDVH) in purple. Bicolor triangles represent sites containing overlapping matches to both motifs. (*b*) Sequence alignment of Paillotin orthologues across diverse *Brachyceran* flies revealed a conserved protein structure, including a signal peptide, a dipeptidyl peptidase cleavage site (XA/XP), and the mature Paillotin peptide. Highlighting corresponds to residues with >50% identity across the alignment.



(e)

Genotype	hpi	Pai peptide abundance (Relative to unchanged)	
		Ecc15	*M. luteus*
w^{1118}	2h	20.82	2.39
	6h	52.35	50.56
	24h	744.43	53.08
Rel^{E20}	6h	0.76	-
spz^{rm7}	6h	-	98.36

Figure 2: *Paillotin* is regulated by the Imd pathway. Transcript levels of *Paillotin*/*CG10332* in w^{1118}, $Relish^{E20}$, spz^{rm7}, and $Relish^{E20}$, spz^{rm7} flies collected at 8 h, 16h, and 24h post-*E. coli* infection (*a*, *c*) and *M. luteus* infection (*b*, *d*). (*e*) Quantified Paillotin peptide levels of w^{1118}, $Relish^{E20}$, and spz^{rm7} hemolymph upon either *Ecc15* or *M. luteus* infection (data extracted from (Rommelaere *et al.*, 2025)). Gene Expression was measured by qRT-PCR and normalized with w^{1118} UC (unchallenged) of wild-type flies set as a value of 1. Error bars represent SEM. Statistical significance was determined using two-way ANOVA (*p<0.05, ** p<0.01, *** p<0.001, n.s. = not significant, p>0.05).

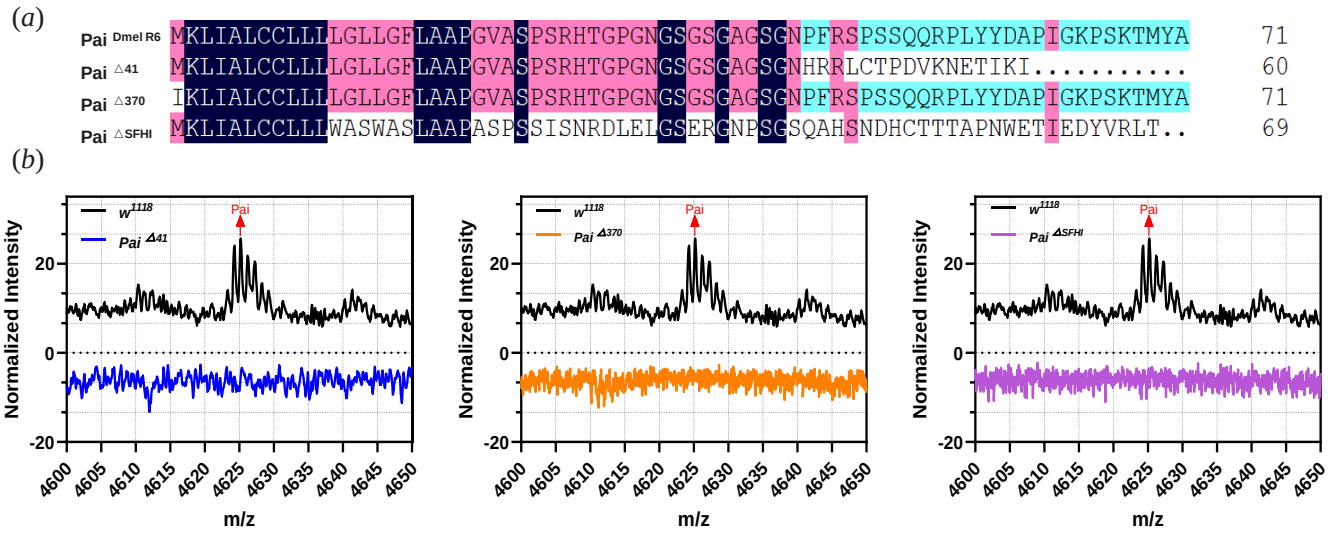


Figure 3: Description and validation of *Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$ mutations. (*a*) Multiple sequence alignment between the Dmel_R6 reference genome Paillotin and Paillotin peptide variants. Conserved residues are highlighted by color coding according to sequence similarity. (*b*) MALDI-TOF proteomic analysis of hemolymph from *w*^{1118}, *Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$ male flies collected 24 hours after septic injury with *Ecc15*.

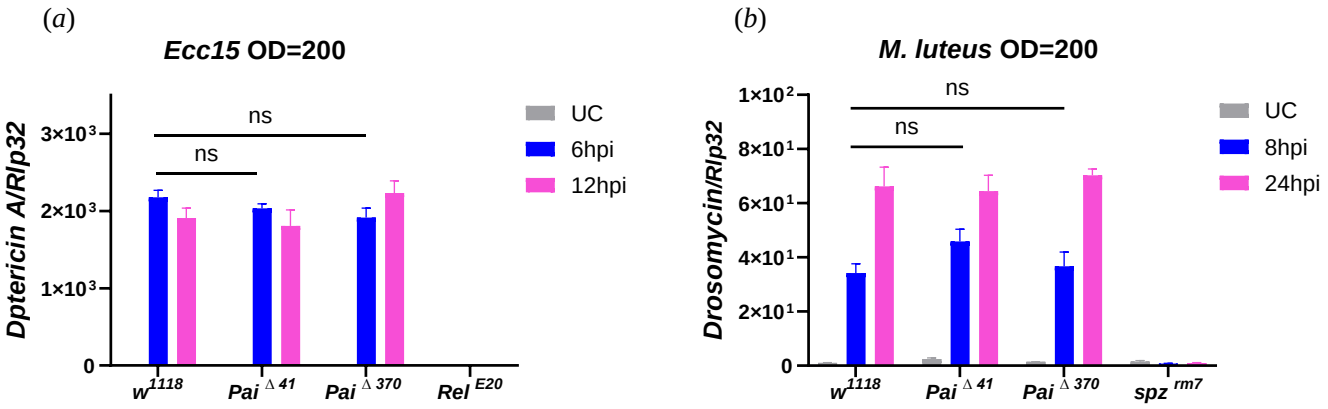


Figure 4: Paillotin does not impact the Imd or Toll pathways. The expression of *Diptericin A* (*a*) at 6 or 12h post-infection with *Ecc15* and *Drosomycin* (*b*) at 8h or 24h post-infection with *M. luteus* were monitored in w^{1118}, $Pai^{\Delta 41}$, and $Pai^{\Delta 370}$ flies. $Relish^{E20}$ and spz^{rm7} were used as the positive control. Expression was normalized with w^{1118} unchallenged (UC) of wild-type set as a value of 1. Error bars represent SEM. Significance was measured by two-way ANOVA (n.s. = not significant, p>0.05).

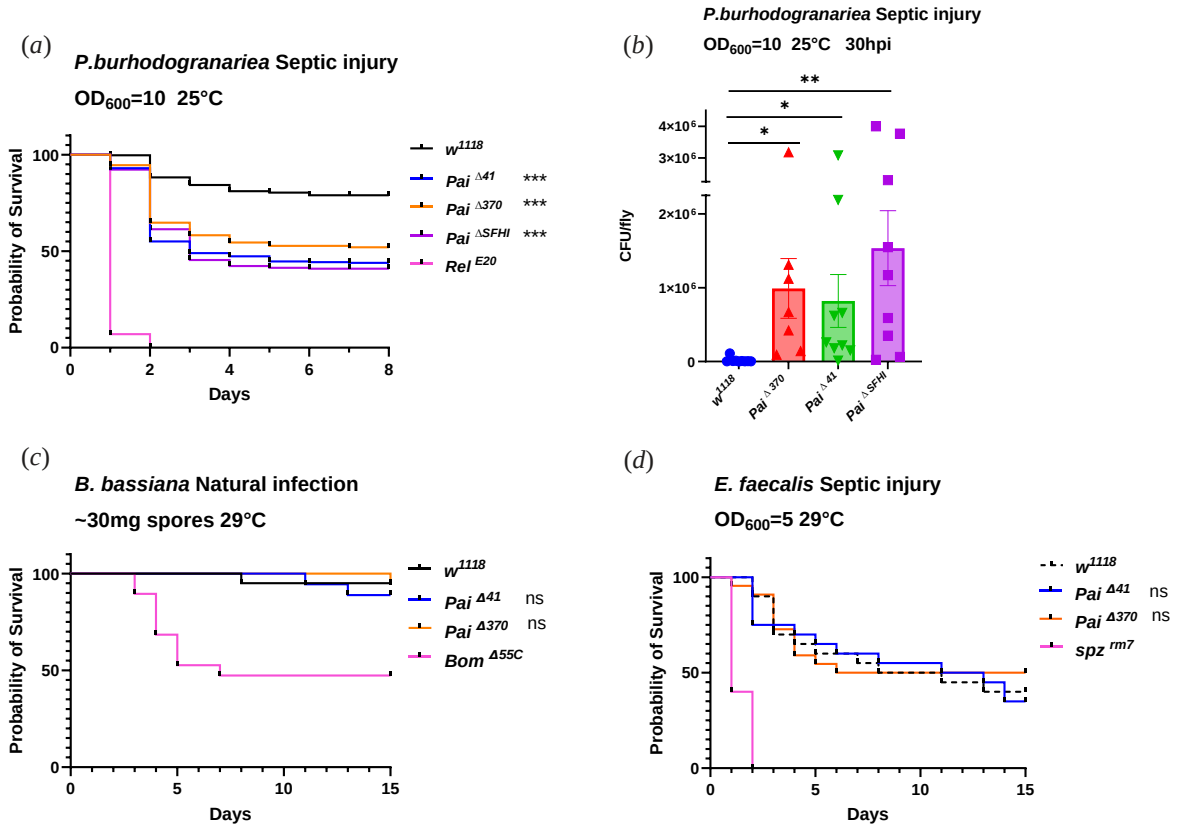


Figure 5: *Paillotin* mutants are susceptible to *P. burhodogranariea* infection. (*a*) The three *Paillotin* mutants exhibit increased susceptibility following septic injury with *P. burhodogranariea* at the indicated bacterial inoculum. Each survival curve represents the pooled results of five independent experiments, with at least 100 flies per genotype. (*b*) Increased bacterial load in *Paillotin* mutants at 30 hpi. Data points represent the mean colony-forming units (CFU) per fly, derived from individual experiments using pools of four flies per genotype following *P. burhodogranariea* infection. No consistent effect of the *Paillotin* mutation on resistance to the Gram-positive bacterium *Enterococcus faecalis* (*c*) or the fungus *Beauveria bassiana* (*d*). Survival curves were analyzed using the Kaplan-Meier, and CFU comparisons were performed using unpaired *t*-tests. Statistical significance is indicated relative to the w^{1118} (*p<0.05, ** p<0.01, *** p<0.001, n.s. = not significant, p>0.05).

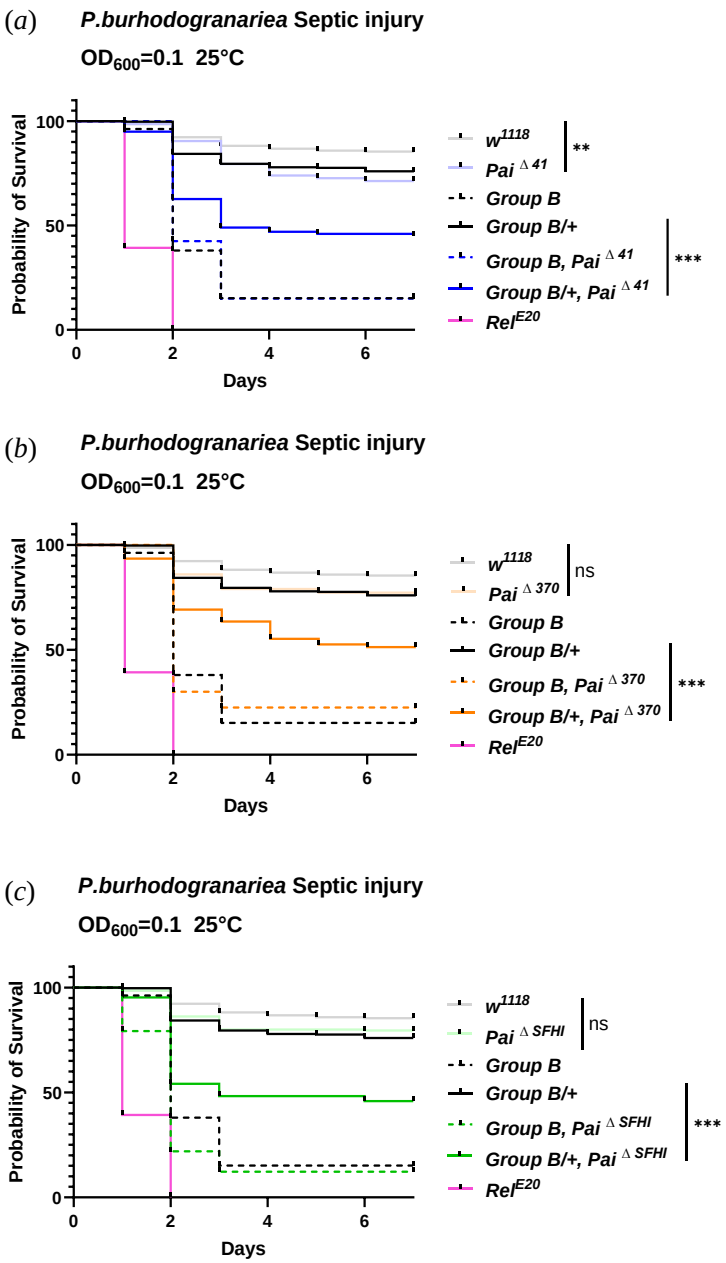


Figure 6: Paillotin synergistically contributes to defense against *P. burhodogranariea* with other Imd-regulated AMPs. (*a-c*) Survival curves showing the synergistic effects between different *Paillotin* mutants ($Pai^{\triangle 41}$, $Pai^{\triangle 370}$, $Pai^{\triangle SFHI}$) and *Group B* mutations (*Drosocin*, *Attacin A, B, C,* and *Diptericin A, B*) in the heterozygous background (*Group B/+*). Survival curves were analyzed using the Cox proportional hazards (CoxPH) model in R version 3.6.3. (** $p<0.01$, *** $p<0.001$, n.s. = not significant, $p>0.05$).

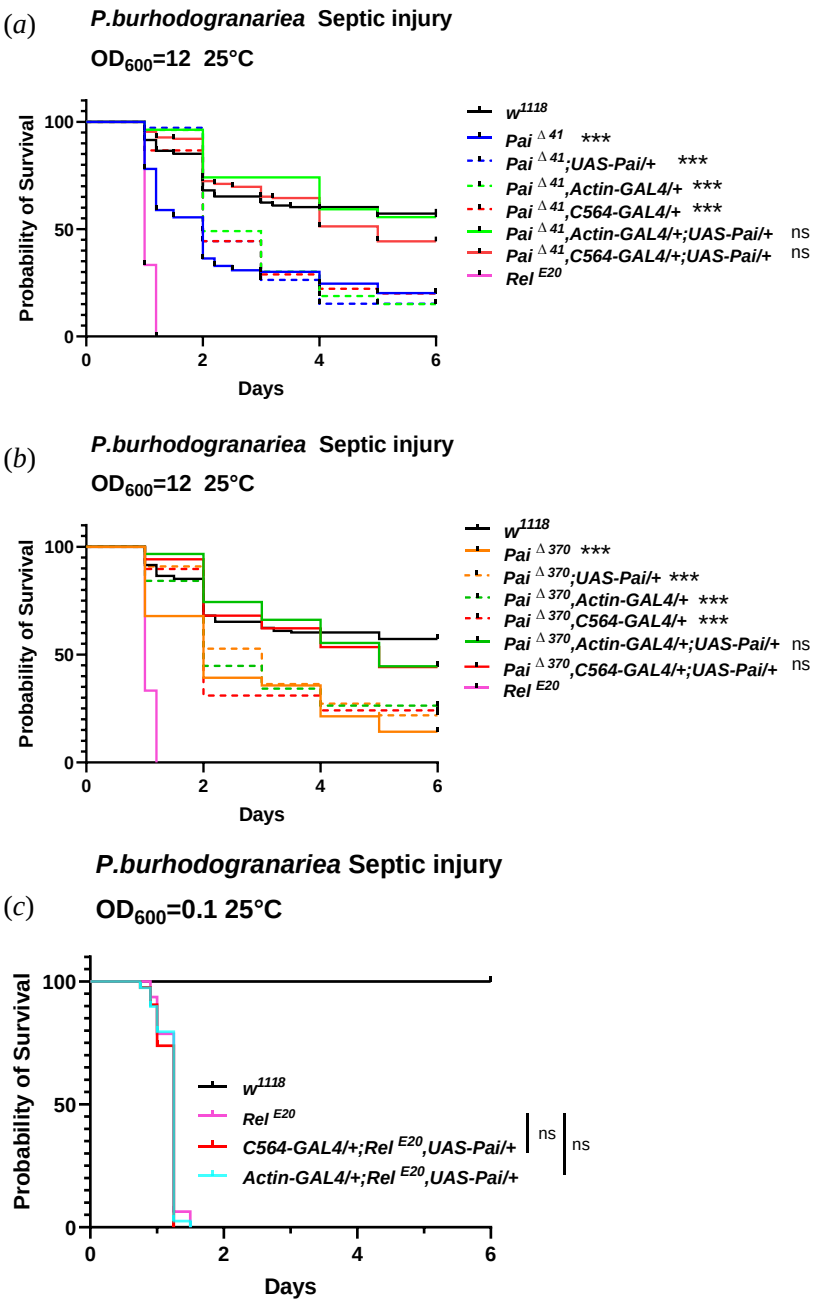


Figure 7: Over-expression of *Paillotin* rescues the resistance of *Paillotin*-deficient flies against *P. burhodogranariea*. (*a*-*b*) Overexpression of *Paillotin* via a combination of the *C564-Gal4* or *Actin-Gal4* and *UAS-Pai* constructs enhances the resistance of the Paillotin mutant to *P. burhodogranariea* infection. (*c*) Overexpression of *Paillotin* in the *Relish*^{E20} background failed to restore resistance. Survival curves were analyzed using the Kaplan-Meier method. (*** $p<0.001$, n.s. = not significant, $p>0.05$).

Supplementary material

Table S1: Collectively, 12 of the 205 *Drosophila* Genetic Reference Panel lines encode a loss-of-function mutation in *Paillotin*. Lines encoding either *Pai*$^{\triangle 41}$ or *Pai*$^{\triangle 370}$ are annotated, alongside the genetic description of each allele.

Mutation	Allele	Reference allele	Alternate allele	Effect	Present in
Pai$^{\Delta 41}$	2R_19488505_DEL	(C)CAAT CGGAG CGTCG TAGTA CAGTG GTCGT TGCTG TGAGC TTGGA GACCT GAACG GATTT CC	(C)-	61bp deletion	28, 41, 176, 227, 321, 406, 491, 852, 879, 892, 911
Pai$^{\Delta 370}$	2R_19488690_SNP	C	T	loss of start codon	370

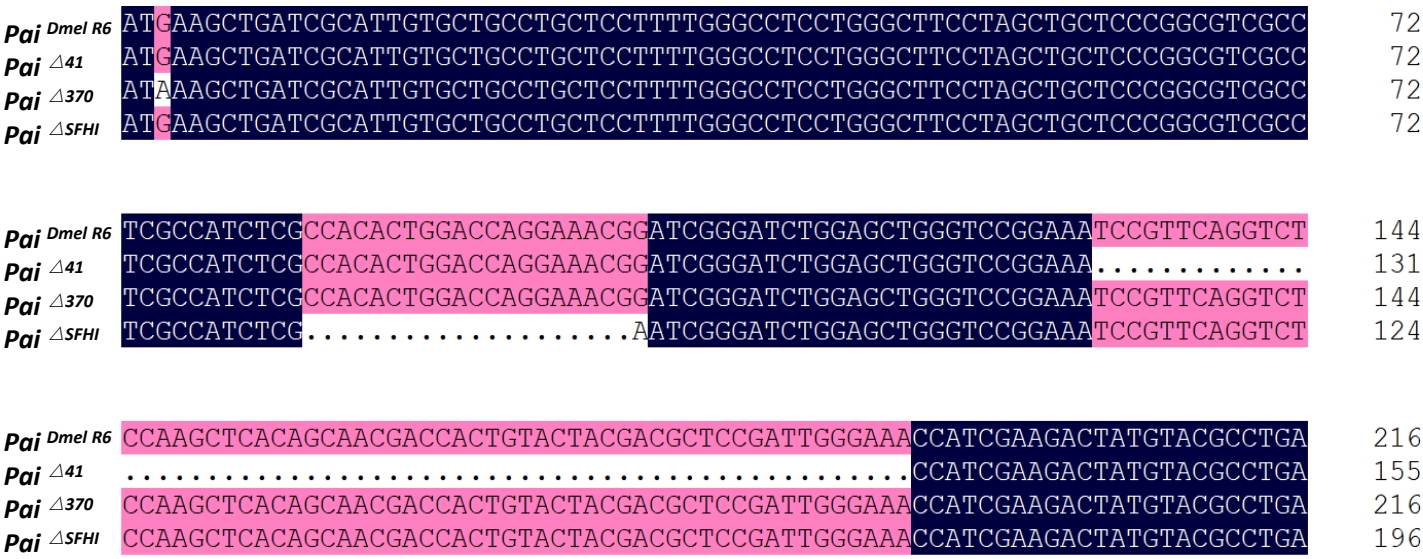


Figure S1. Nucleotide alignment of wild-type and mutant *Paillotin* alleles. Nucleotide sequences of wild-type *Paillotin* (*Pai*$^{Dmel\ R6}$) and the three mutant alleles (*Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$) used in this study are aligned.

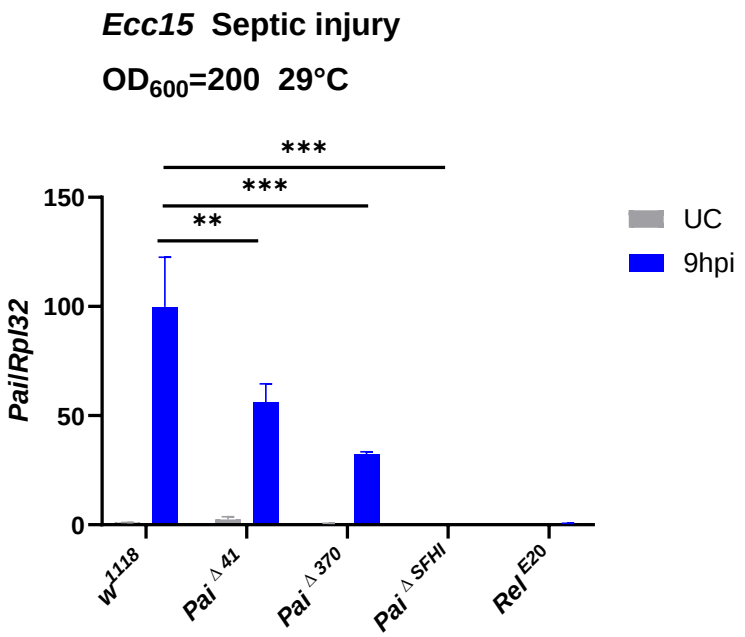


Figure S2: Expression of *Paillotin* in *Pai*$^{\triangle 41}$, *Pai*$^{\triangle 370}$, and *Pai*$^{\triangle SFHI}$ mutations.

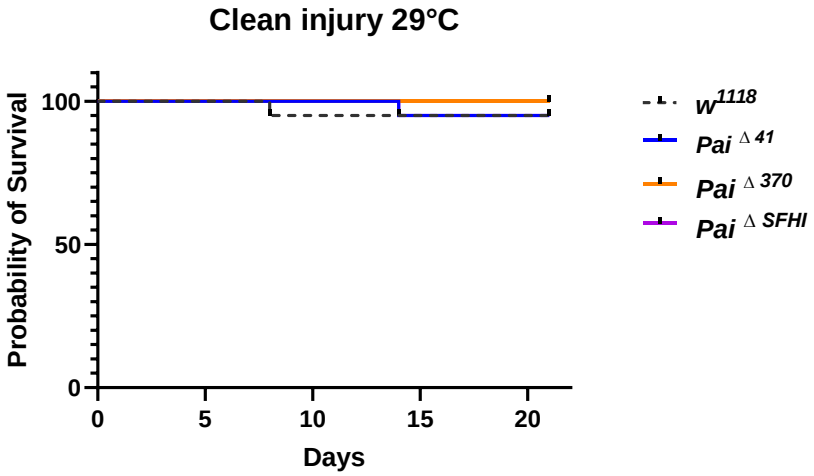


Figure S3: No susceptibility of *Paillotin* mutants to clean injury. The thorax of wild-type and mutants was pricked with a clean needle.

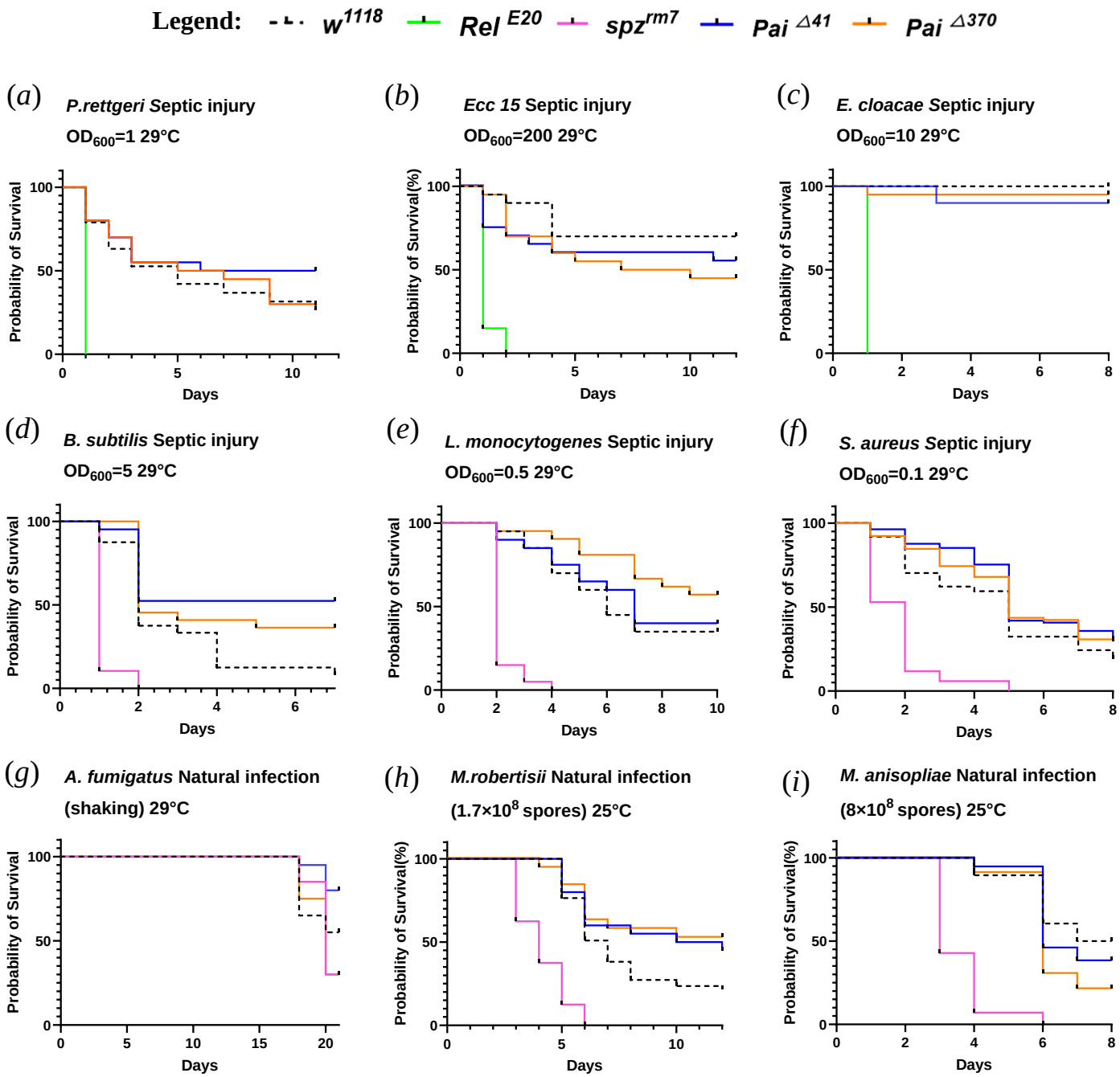


Figure S4: No consistent effect of *Paillotin* mutation on resistance to a broad spectrum of microbes. w^{1118} flies were utilized as wild-type controls, while spz^{rm7} flies lack the Toll pathway, and $Relish^{E20}$ flies lack the Imd pathway, were used as susceptible models for all survival experiments against infections. Tm and OD600 are indicated.

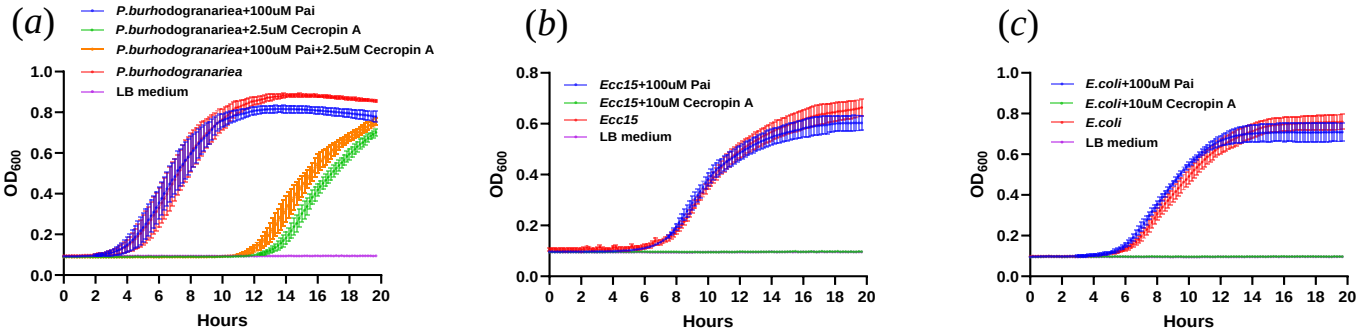


Figure S5: Paillotin did not show microbicidal activity *in vitro*. (*a*) The growth of *P. burhodogranariea* was monitored in the presence of the Paillotin peptide (100 µM) and/or the Cecropin A peptide (2.5 µM). The growth of *Ecc15* (*b*) and *E. coli* (*c*) was monitored in the presence of the Paillotin peptide (100 µM). Optical density (OD) values were measured every 10 minutes over 20 hours to generate bacterial growth curves. Different colored curves represent the conditions tested, as indicated in the figure legend.

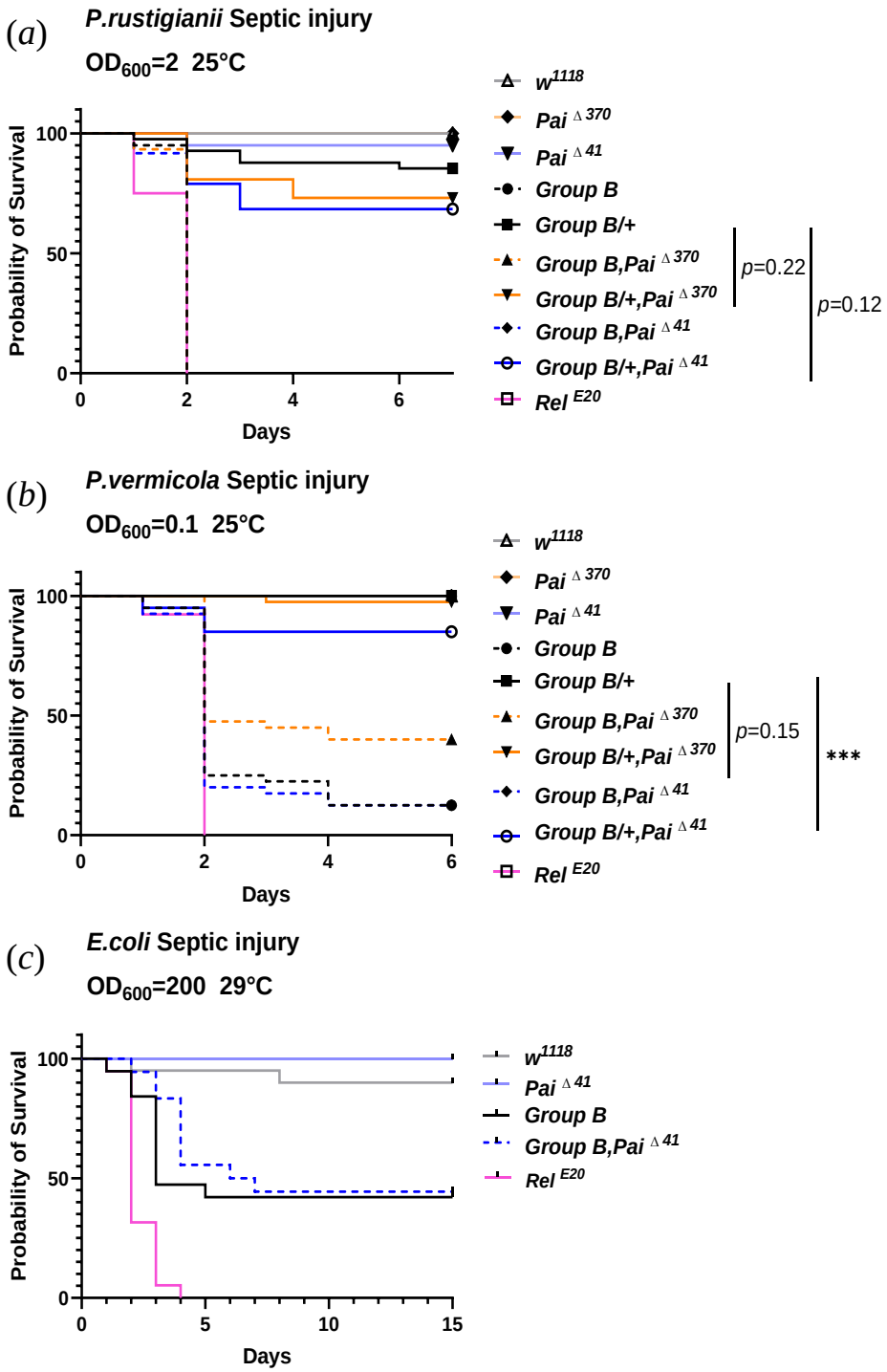


Figure S6: *Paillotin* shows a minor synergistic effect with *Group B* mutants against other *Providencia* species at the doses tested. (*a*) Both *Group B/+*, *Pai* compound mutants suffer a minor increase in mortality compared to *Group B/+* alone upon *Providencia rustigianii* infection. Although the synergistic effect between Paillotin and Group B was only marginally significant (Mutant, Cox Hazard Ratio (HR), *p*-value: *Paillotin*, HR =-0.01, *p*=0.904; *Group B*, HR =-2.8, *p*<0.001; *Paillotin***Group B*, HR = -0.41, *p*=0.09). (*b*) *Group B/+*, *Pai*$^{\Delta 41}$ mutants suffer a mortality increase compared to *Group B/+*. In addition, *Group B/+ Pai*$^{\Delta 370}$ shows no difference from *Group B/+*, but also *Group B, Pai*$^{\Delta 370}$ compound mutants survive better than *Group B* alone, indicating a complex phenotype after recombination for this infection that may mask the contribution of *Pai*$^{\Delta 370}$ at the chosen low-mortality dose (Mutant, Cox Hazard Ratio (HR), *p*-value: *Paillotin*, HR =0.15, *p*=0.14; *Group B*, HR =-2.7, *p*<0.001; *Paillotin***Group B*, HR = -1.13, *p*=0.03). Differences between *Pai*$^{\Delta 41}$ (truncated) and *Pai*$^{\Delta 370}$ (loss of start) may explain this difference. (*c*) No survival difference was observed for *Group B, Pai* compound mutants against *E. coli*.

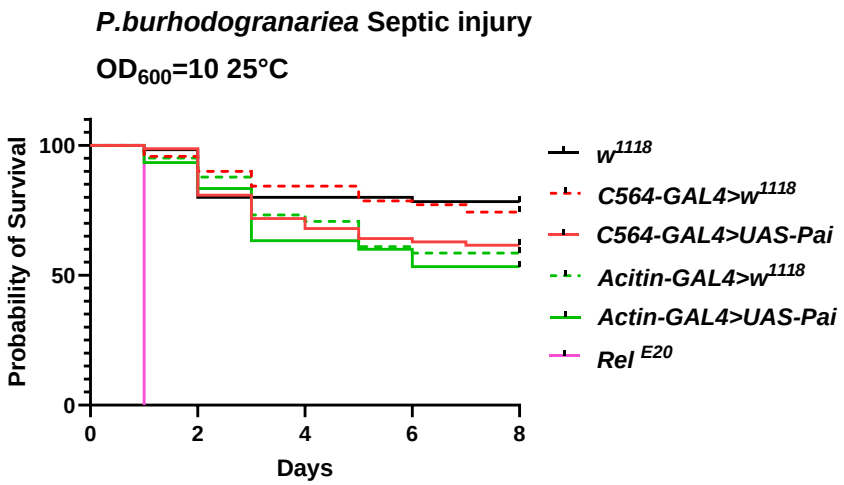


Figure S7: Overexpression of *Paillotin* does not confer protection against *P. burhodogranariea* infection in wild-type flies. Survival curves of flies overexpressing *Paillotin* by using *C564-Gal4* and *Actin-Gal4* drivers following septic infection with *P. burhodogranariea*.

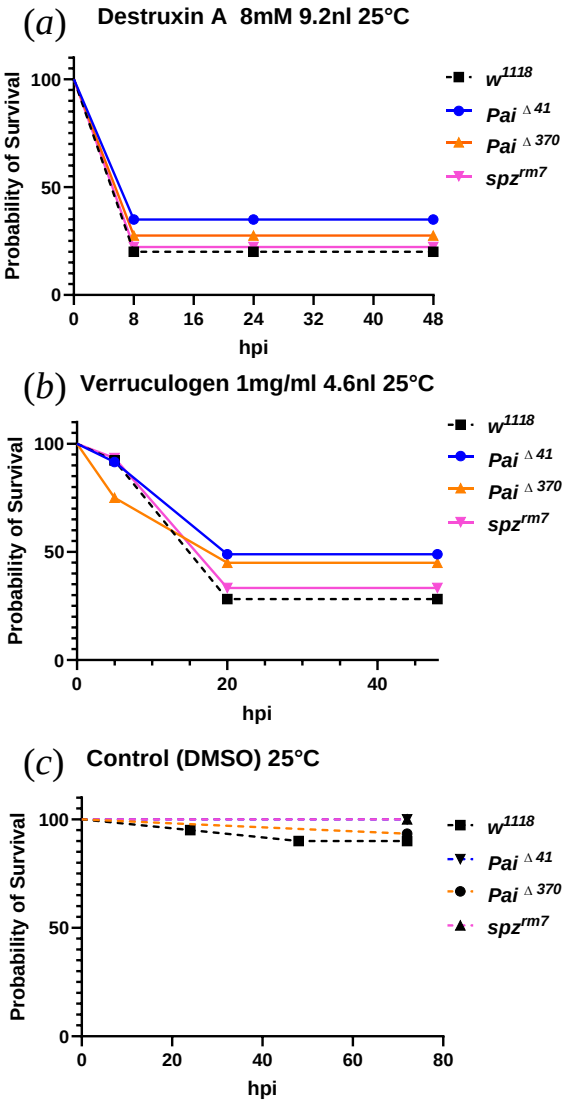


Figure S8: Paillotin does not contribute to protection against fungal toxins. (*a*) Survival of wild-type and *Paillotin* mutant flies following injection with 9.2 nl of 8 mM Destruxin A, a mycotoxin secreted by *M. robertsii*. (*b*) Survival of wild-type and *Paillotin* mutant flies injected with 4.6 nl of 1 mg/ml Verruculogen, a neurotoxin produced by *A. fumigatus*. (*c*) Survival of all tested fly strains following injection with DMSO as vehicle control. spz^{rm7} flies are used as positive controls.

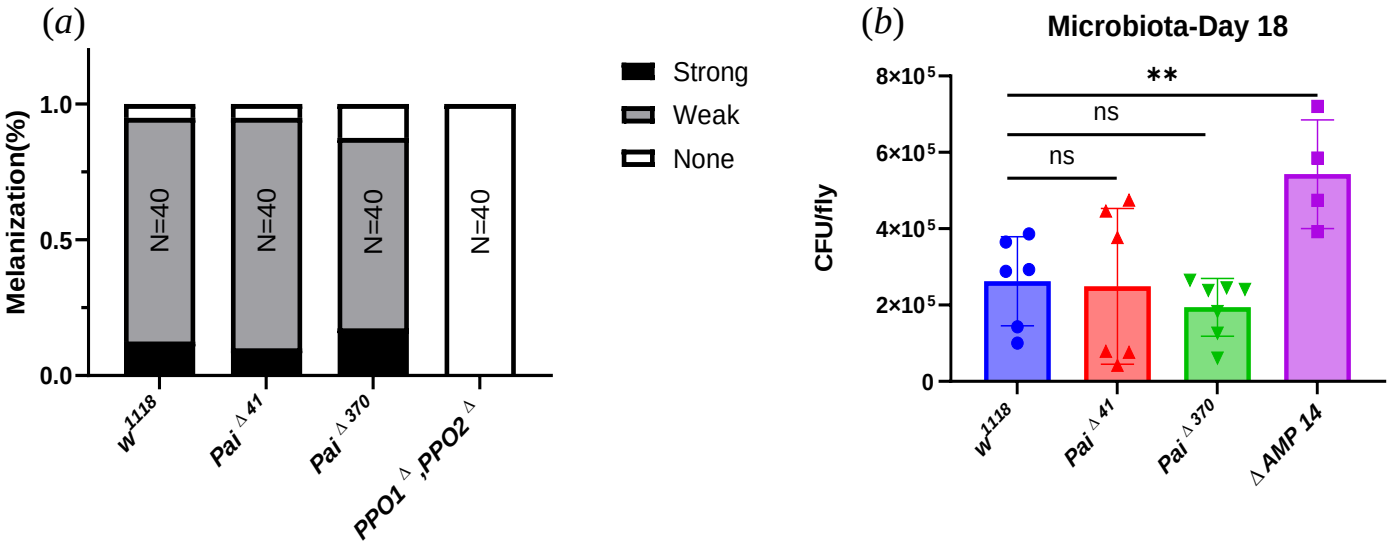


Figure S9: Paillotin is not involved in melanization response and microbiota regulation. (*a*) Cuticle blackening after clean injury is not impaired except in melanization-deficient flies (*PPO1*$^{\triangle}$, *PPO2*$^{\triangle}$). (*b*) No significant difference in microbiota load between *Paillotin* mutants and wild-type flies. △ *AMP14* is used as the positive control.

Table S2: Drosophila strains used in this study.

Drosophila lines	Reference
iso; iso; Rel^{E20}	Hedengrenetal.,1999
iso; iso; spz^{rm7}	Lemaitre et al.,1996
iso; Bom$^{\triangle 55}$; iso	Hanson et al., 2019
iso; iso; Rel^{E20}, spz^{rm7}	Ryckebusch et al., 2025
iso; Pai$^{\triangle 41}$; iso	DGRP
iso; Pai$^{\triangle 370}$; iso	DGRP
; Pai$^{\triangle SFHI}$;	Guangzhou Drosophila Resource Center
; ; UAS-Pai	This paper
iso ;Group B; iso	Hanson et al.,2019
iso ; PPO1$^{\triangle}$,PPO2$^{\triangle}$; iso	Bingelli et al., 2014
△AMP 14	Carboni et al., 2022
C564-Gal4	BDSC
Actin-Gal4	BDSC

Table S3: Microbial strains used in this study.

Type	Species	Strain,Source, DOI of previous study
Gram-negative	*Escherichia coli*	1106
	Pectobacterium carotovorum	Ecc15
	Providencia rettgeri	Dmel
	Enterobacter cloacae	B12
	Providencia burhodogranariea	B
	Providencia rustigianii	DSM 4541
	Providencia vermicola	DSM 17385
Gram-negative	*Micrococcus luteus*	doi:10.7554/eLife.44341
	Bacillus subtilis	168
	Enterococcus faecalis	254
	Listeria monocytogenes	BUG 1600
	Staphylococcus aureus	doi:10.7554/eLife.44341
Fungi	*Beauveria bassiana*	R444
	Metarhizium anisopliae	ARSEF-2575
	Metarhizium robertsii	ARSEF-2575
	Aspergillus fumigatus	doi:10.7554/eLife.44341